IDENTIFICATION OF A PLASTOQUINONE AND TWO NAPHTHOQUINONES IN *ANACYSTIS NIDULANS* BY NMR AND MASS SPECTROSCOPY[1]

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A comparative study of quinones in photosynthetic plants by Crane and co-workers has included *Anacystis nidulans*, a blue-green alga. Henninger, Bhagavan and Crane (1965) found three quinones of undetermined structure: a plastoquinone, a "hydroxynaphthoquinone," and a "polar naphthoquinone." The plastoquinone seemed similar to plastoquinone A (45), but differed slightly in R_f values on thin-layer chromatography and was more prone to decomposition on isolation. The hydroxynaphthoquinone running just above the plastoquinone on thin-layer chromatography resembled vitamin K_1 in chromatography and ultraviolet absorption, but showed strong infrared absorption indicating the presence of a hydroxyl group. The second naphthoquinone was considered more polar than vitamins K_1, K_2 or K_3 as it had lower R_f values on silicic acid chromatography. We have determined that these compounds are, respectively, plastoquinone A (45) (2,3-dimethyl-5-nonaprenyl-1,4-benzoquinone), vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), and a monohydroxy analog of vitamin K_1 in which the hydroxyl substituent is at position b or c in the phytyl side chain (Structure I). In accord with the result from Crane's laboratory, no tocopherol was found.

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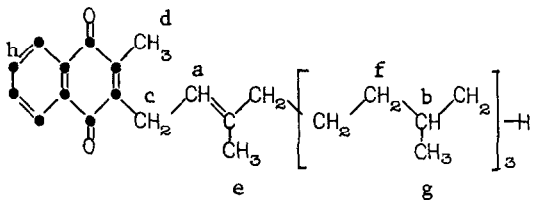


I (letters refer to NMR assignments, Table II)

EXPERIMENTAL

Anacystis nidulans, obtained from J. Myers, University of Texas, was grown in 20 liter batches in a synthetic medium under 5% CO_2 at 30°C with illumination from fluorescent lights. Algae were harvested in a Sharples centrifuge and the lipids extracted at once under a CO_2 atmosphere in subdued light. In a typical extraction, 100 g of wet packed cells were extracted with 600 ml of chloroform-methanol (1:2 by volume) followed by 200 ml of chloroform. The aqueous phase formed upon mixing 200 ml of water with the combined extracts was discarded. The chloroform phase was evaporated at 40° under vacuum. Lipid residue (3.5 g) was taken up in chloroform-methanol (19:1 by volume) and filtered prior to chromatography.

Isolation of Quinones.- The lipid extract was chromatographed (Allen *et al.*, 1966) on 50 g of DEAE cellulose (acetate form) in a 2.5 cm ID column to remove the more polar lipids which were desired for other studies. A fraction containing quinones, pigments, chlorophyll, and galactolipids was eluted with chloroform-methanol (19:1 by volume), and the solvent evaporated at 40° in a vacuum rotary evaporator. The residue taken up in 25-30 ml of heptane was applied to a 2.5 cm ID column of 40 g of silicic acid (Merck, Darmstadt, 0.05-0.2 mm mesh) packed in heptane. All operations were carried out in subdued light in a nitrogen atmosphere. Components were eluted as follows.

Eluant	Volume	Eluted Material
Heptane	600 ml	Carotene, wax, yellow unknown
10% benzene/heptane	500 ml	
30% benzene/heptane	600 ml	Vitamin K_1 in 3rd to 5th 45 ml fractions
		Plastoquinone in 6th to 13th 45 ml fractions
60% benzene/heptane	500 ml	
benzene	500-1000 ml	Polar naphthoquinone in approx 13th through 23rd 45 ml fractions

Fractions were monitored by thin-layer chromatography on silicic acid plates developed in chloroform and visualized with a spray of 10% phosphomolybdic acid in ethanol followed by 5 minutes heating at 110°C (blue spots on a yellow background). Appropriate fractions were combined and evaporated at 40° under nitrogen. For additional purification, which was often necessary, the combined fractions were chromatographed as bands on thin layer plates as above, scraped off and eluted with chloroform.

Ultraviolet spectra were determined in 95% ethanol.

Infrared spectra were determined in KBr pellets, or as thin films on NaCl windows.

NMR spectra were run in carbon tetrachloride solution on a Varian A-60 spectrometer with time-averaging computer. Tetramethylsilane served as a trigger in the "NMR trigger mode," or a benzene trigger was used on the "sideband mode" to avoid interference in the region of tetramethylsilane spinning sidebands. Up to 2,000 scans were run as necessary for the amount of material in hand.

Mass spectra were determined with an Hitachi RMU-6D single-focusing instrument at 70 V ionizing potential. The sample was vaporized at the ion source with a heated direct inlet system.

RESULTS

R_f values and ultraviolet absorption maxima of the purified quinones are

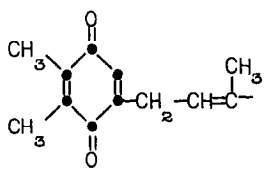
indicated in Table I.

TABLE I

Compound	R_f in chloroform	UV Maxima, mμ (95% ethanol)
Plastoquinone A (45)	(Spinach) 0.72	255
	(*Anacystis*) 0.72	255
Vitamin K_1	(Commercial) 0.71	330, 272, 263, 250, 244
	(*Anacystis*) 0.71	330, 272, 263, 249, 243
Hydroxynaphthoquinone	0.26	335, 272, 265, 249, 245

The plastoquinone was recrystallized from ethanol to give a constant melting point of 47-48°C (micro hot stage), in agreement with the value reported by Henninger *et al.* (1965). It did not show the exceptionally high instability reported, but algae or lipid extracts stored for several days even in liquid nitrogen lost quinone steadily and decomposition products made chromatographic purification more difficult.

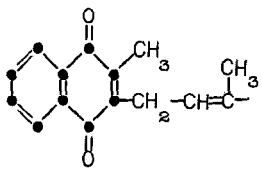
Identification of Plastoquinone A (45).- Mass spectra run at a spectrometer pressure of 2×10^{-7} Torr and 330-390° sample temperature showed an intense parent peak at M/e = 748 corresponding to $C_{53}H_{80}O_2$. Other peaks which stand out correspond to loss of CH_3 (733), 1 to 6 C_5H_8 (isoprene) units (M/e = 680, 612, 544, 476, 408, 340), and 1 to 4 isoprene units plus a methyl (M/e = 665, 597, 529, 461). As reported by Das *et al.* (1965) for the plastoquinone series, the base peak occurs at 189 and corresponds to the fragment



which confirms the presence of the 2,3-dimethyl-1,4-benzoquinone nucleus. We did not find an enhanced M + 2 peak reported by Das. NMR absorption bands of appropriate area were observed at τ = 3.50, 4.83, 6.80, 6.93, 8.00 and 8.40 in accord with the data reported by Planta *et al.* (1959) and by

Eck and Trebst (1963) for plastoquinone A (45). The infrared spectrum was similar to the one published by Kofler *et al.* (1959).

Identification of Vitamin K_1.- The mass spectrum of the *Anacystis* naphthoquinone of R_f 0.71 was identical in all significant features to that of a commercial sample of vitamin K_1 (Calbiochem, Los Angeles, California). Adequate spectra were obtained at 60-70° sample temperature and 2 x 10^{-7} Torr. The parent peak was at M/e = 450 ($C_{31}H_{46}O_2$) and was also the base peak. The next most intense peak at M/e = 225 would correspond to $C_{15}H_{13}O_2$ probably originating from the fragment



in analogy to the fragmentation of plastoquinones. An M-15 peak is also prominent, but the saturated phytyl side chain did not give prominent peaks corresponding to loss of C_5 units as did the polyprenyl side chain of plastoquinone.

The NMR and infrared spectra of the *Anacystis* quinone and corresponding spectra of authentic vitamin K_1 were also essentially identical.

The polar naphthoquinone (R_f 0.26) of *Anacystis* is shown to be a hydroxynaphthoquinone $C_{31}H_{48}O_3$ by mass and infrared spectra. The mass spectrum (sample at 170°, 2 x 10^{-7} Torr) has the base at M/e = 225 suggesting a fragment identical to that from vitamin K_1. Peaks at 197 and 240 were nearly as intense as the base peak. A strong parent peak at M/e = 466 was a little less intense than the M-18 peak suggesting facile loss of water. An M-33 peak (loss of CH_3 + H_2O) is the only other prominent peak in the high mass range. These results suggest hydroxyl substitution on the phytyl side chain.

NMR spectral data is summarized in Table II.

TABLE II

NMR τ Values of Absorption Peaks

Assignment (see Structure I)	Vitamin K_1 (Authentic) (100 scans)	Hydroxynaphthoquinone (*Anacystis*) (2,000 scans)
a	5.00 (triplet)	4.9 (unresolved multiplet)
hydroxyl group	-	6.4 (broad)
c	6.72 (doublet)	6.74 (doublet)
d	7.90	7.90
e	8.25	8.25
b, f	8.82	8.75
g	9.15 (doublet)	9.15 (doublet)
h	2.08, 2.42 (multiplets)	2.02, 2.36 (multiplets)

No absorption which could be attributed to a O-C-H proton was observed; in particular, there was no absorption in the τ = 5.0 to 6.5 region which suggests absence of the OH on the methylene carbons f and the methyl carbons d, e and g. This leaves the possibility of substitution on one of three tertiary carbons b, or on the methylene c if the questionable assumption is made that substitution of an oxygen on this already de-shielded carbon would not shift the peak appreciably. The uncertainty in position could be resolved from integration of peak areas, but detection of a one proton difference is impossible in the τ = 8 to 9 region.

In vitamin K_1 relative peak areas a:c were found to be 1.0:1.9 (theory 1:2) but in the hydroxynaphthoquinone relative areas a:b:c were 1.0:1.0:1.2 (theory for -OH at c 1:1:1) which would suggest substitution at c.

CONCLUSION

Addition of NMR and mass spectrometry to the techniques conventionally used for analysis of biological quinones makes possible unequivocal identification of small quantities ($\sim$ 1 mg) of material such as may be isolated by

preparative thin-layer chromatography.

In *Anacystis nidulans* the method has demonstrated the presence of plastoquinone A (45), vitamin K_1, and a hydroxy substituted vitamin K_1 with the single hydroxyl probably located on a tertiary carbon of the phytyl side chain, or possible on the α-carbon.

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